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I, ANNA MAIJA MADL, ACTING TEAM LEADER EXAMINATION SUPPORT & SALES hereby certify that annexed is a true copy of the Provisional specification in connection with Application No. PP 8131 for a patent by HYAL PHARMACEUTICAL AUSTRALIA LIMITED filed on 13 January 1999.

I further certify that the name of the applicant has been amended to MEDITECH RESEARCH LIMITED pursuant to the provisions of Section 104 of the Patents Act 1990.

COMMONWEALTH OF AUSTRALIA
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Twenty-second day of February 2000

A. M. Madl

ANNA MAIJA MADL
ACTING TEAM LEADER
EXAMINATION SUPPORT & SALES

PRIORITY DOCUMENT
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AUSTRALIA

Patents Act 1990

PROVISIONAL SPECIFICATION

Applicant(s): Meditech Research limited.
~~HYAL PHARMACEUTICAL AUSTRALIA LIMITED~~

Invention Title:

A COMPOSITION AND METHOD FOR THE ENHANCEMENT OF THE EFFICACY OF DRUGS

The invention is described in the following statement:

of the cancer cells to the active compound is often observed. Moreover, it is often found that resistance to one drug may confer resistance to other biochemically distinct drugs. This has been termed multidrug resistance. Drugs that are typically affected by the multidrug resistance problem include doxorubicin, vincristine, vinblastine, colchicine and actinomycin D. In at least some cases, multidrug resistance is a complex phenotype which has been linked to a high level of expression of a cell membrane drug efflux transporter called MdrI protein, also known as P-glycoprotein. This membrane "pump" has broad specificity, and acts to remove from the cell a wide variety of chemically unrelated toxins (see Endicott et al., 1989).

Recently, a similar mechanism of broad spectrum drug resistance has been reported for certain microorganisms. These results indicate the existence of bacterial efflux systems of extremely broad substrate specificity that are similar to the multidrug resistance pump of mammalian cells (see Nikaido, 1993).

Substances which reverse multidrug resistance are known as resistance modification agents (RMAs), and are of importance in potentiating the cytotoxicity of chemotherapeutic agents to which a human cancer has become resistant. Although many agents have been identified as RMAs *in vitro*, a large proportion of these have little or no therapeutic potential because of high toxicity *in vivo* at the doses required to reverse multidrug resistance. For example, metabolic poisons, such as azide, are able to reverse multidrug resistance *in vitro*, but have no usefulness *in vivo*. Most other highly effective RMAs, such as PSC833, appear to work as competitive antagonists of a drug binding site on the MdrI protein. Many of these agents also have toxicity which limits their usefulness *in vivo*. Consequently, there is a need to develop alternate pharmacological strategies for reversing multidrug resistance.

because methotrexate is relatively insoluble in water; therefore if this were to occur it would be a very weak interaction. The most likely bonding between MTX and HA would be via hydrophobic interactions between MTX's numerous hydrophobic groups and the hydrophobic patches in the secondary structure of HA(Scott et al., 1989).

(ii) *Molecular association*

Where MTX is merely "mixed" in HA gel (Figure 2B) with no specific chemical bond formation, MTX could become entrapped within the 3-dimensional meshwork formed by higher concentrations of HA (Mikelsaar and Scott, 1994), so that the drug simply diffuses from the HA after administration. If HA is rapidly taken up and bound by specific cell receptors, the drug will be released in higher concentration at these points eg. lymph nodes, liver, bone marrow, tumour cells with HA receptors.

While again not wishing to be bound by any particular theory, one mechanism by which HA helps to target active agents may be via the characteristic over-expression of HA receptors in several tumour types (Stamenkovic *et al.*, 1991; Wang *et al.*, 1998). The HA receptors CD44, Receptor for Hyaluronan Mediated Motility (RHAMM) and ICAM-1, have been linked to tumour genesis (Bartolazzi *et al.*, 1994) and progression (Günthert 1993; Arch *et al.*, 1992). RHAMM is a major factor in mediating tumour cell motility and invasion (Hardwick *et al.*, 1992). It has been demonstrated that RHAMM is required for H-ras transformation of fibroblasts (Hall *et al.*, 1995), which would make this receptor a potential participant in tumour formation and growth. ICAM-1, a receptor tentatively linked to HA metabolism (McCourt *et al.*, 1994), is highly expressed in transformed tissues such as mouse mastocytomas (Gustafson *et al.*, 1995) and in the stroma and clusters of tumour cells of human breast carcinomas (Ogawa *et al.*, 1995).

Increased expression of HA receptors on tumour cells provides a rationale for attempting the incorporation

effectiveness of a drug, comprising the step of administration of a pharmaceutical composition comprising hyaluronan and said drug.

While not wishing to be bound by theory, it is believed possible mechanisms for overcoming drug resistance are:

1. Hyaluronan binds to receptors on the resistant cell or enters the cell via bulk endocytosis, resulting in the entrained or bound drug being delivered into the cell, allowing it to become therapeutically active.

2. Hyaluronan binds to the surface of the resistant cell, where the entrained or bound drug diffuses from the hyaluronan meshwork into the cell, resulting in the drug being delivered to the resistant cell.

3. Hyaluronan and other mucopolysaccharides adopt a coiled configuration which entrains the drug, and may also bind a variety of drugs.

Accordingly, in a fourth aspect of the present invention there is provided a method of overcoming drug resistance, comprising the step of co-administering a drug with a mucopolysaccharide capable of entraining and/or binding said drug and capable of binding to receptors on the resistant cell or entering the cell via bulk endocytosis, wherein said drug is delivered into the cell, thereby allowing it to become therapeutically active.

According to a fifth aspect of the present invention there is provided a method of overcoming or reducing drug resistance, comprising the step of co-administering a drug and a mucopolysaccharide capable of entraining and/or binding said drug and capable of binding to the surface of the resistant cell, wherein the entrained or bound drug diffuses from said mucopolysaccharide into the cell.

While not wishing to be bound by theory, it may also be that a combination of hyaluronan with a drug results in the drug being retained in the cell for a longer period, allowing a prolonged release and more time for the

co-administering a drug and a mucopolysaccharide capable of entraining or binding said drug and/or associating with said drug in such a manner that said drug has reduced gastrointestinal toxicity.

Throughout the description and claims of this specification, the word "comprise" and variations of the word, such as "comprising" and "comprises", means "including but not limited to" and is not intended to exclude other additives, components, integers or steps.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows that in higher concentrations HA forms a three-dimensional meshwork which is capable of entraining small molecules such as methotrexate. The HA/drug targeting of pathological sites is accomplished by the HA rapidly binding to specific cell receptors, followed by diffusion of the drug from the HA, and/or co-internalization of both the HA and drug via HA and/or drug receptors.

Figure 2A shows the possible molecular interactions between methotrexate and HA. These include (i) ionic bonding, (ii) hydrogen bonding or (iii) hydrophobic bonding.

Figure 2B is a diagrammatic representation of the entanglement of methotrexate in HA. At higher concentrations HA forms a 3-dimensional meshwork which is represented by the large coiled molecule. (*) represents the methotrexate which has a molecular weight of only 454D, and is easily entrained in the 400-900 kD HA molecule.

Figure 3 shows the general pathology of human breast cancer tumours grown in nude mice. Panel A shows the general morphology of a grade II-III human tumour. Panel B shows a micrograph of another section of the tumour exhibited in Figure 3A. This section shows the surrounding mouse muscle (M), tumour capsule (C), necrotic areas of the tumour (N), infiltrating tumour (T) and (→) indicates a common phenomenon known as "Indian files", in which

DETAILED DESCRIPTION OF THE INVENTION

The invention will now be further described by way of reference only to the following non-limiting examples. It should be understood, however, that the examples following are illustrative only, and should not be taken in any way as a restriction on the generality of the invention described above. In particular, while the invention is described in detail in relation to cancer, it will be clearly understood that the findings herein are not limited to treatment of cancer. For example, cytotoxic agents may be used for treatment of other conditions; methotrexate is widely used for treatment of severe rheumatoid arthritis.

Example 1 Validation of human breast cancer tumours in nude mice and identification of hyaluronan receptors on the breast tumours *in situ*

To establish an appropriate animal model for human breast cancer, it was necessary to perform pathological testing. For a tumour to be physiologically viable neovascularization is essential, because the capillary network supplies nutrients to the tumour. The presence of vascularisation, ductal invasion, necrosis, apoptosis, a high mitotic index and nuclear abnormalities are all characteristic of breast carcinoma.

The human breast carcinoma cell line MDA-MB-468 (American Tissue Culture Collection, Rockville, U.S.A) was selected on the basis of its expression of the HA receptors, CD44, RHAMM and ICAM-1. Cells were routinely grown and subcultured as a monolayer in 175cm^2 culture flasks in Leibovitz L-15 Medium supplemented with 10% foetal calf serum (FCS) and 10µg/ml gentamicin. For injection into mice cells were grown to 100% confluency, trypsinised in 0.05% trypsin/0.01% EDTA solution, washed twice by centrifugation in a Beckman TJ-6 bench centrifuge (Beckman, Australia) at 400*g*av for 10min, counted using a Model-ZM Coulter counter (Coulter Electronics, England),

rinse. The detection antibodies were applied for 60min at room temperature (RT). The antisera or antibodies were directed against RHAMM (Applied Bioligands Corporation (Manitoba, Canada), ICAM-1, CD44v6, CD44v10, total CD44H, and CAE. All other detection antibodies were purchased from Zymed (California, U.S.A). The slides were washed 3x5min in PBS and endogenous peroxidase activity blocked by immersion in 0.3%H_2O_2/ methanol for 20min. Following a further PBS wash, peroxidase-conjugated pig anti-rabbit secondary antiserum (Dako, Denmark) was applied for 60min at RT, followed by 3x5min wash in PBS. Sigma *Fast* DAB (3,3'-Diaminobenzidine, Sigma, St. Louis, U.S.A) tablets were prepared according to the manufacturer's instructions and the DAB solution was applied for 5-10min at RT. The slides were washed in tap water for 10min, counterstained with haematoxylin, dehydrated and mounted.

Examination of the haematoxylin and eosin-stained breast tumour sections demonstrated all of the usual features associated with viable tumours, as shown in Figures 3 and 4, confirming that the animal host successfully maintained a grade II human breast carcinoma. There are several features which are characteristic of malignancy. The section of the slides labelled (B) displays these features. All of the pathological features of malignancy observed are in section (B), ie

i) high nuclear/cytoplasmic ratios

ii) angular chromatin and nucleoli

iii) irregular nuclear membrane

It was concluded that a grade II-III level tumour was able to be supported in the nude mouse model. A grade II-III level tumour generally gives a prognostic survival rate of about 47% (Bloom and Richardson, 1957). A grade II-III level tumour is characterised by:

i) moderate nuclear pleomorphism, hyperchromatin, and mitotic activity, features observed throughout the displayed section of tumour; and

ii) little or no duct formation.

Table 1: Expression of hyaluronan receptors on human breast tumour xenografts

The rating index for the percentage of epitope expression on the tumour was quantitated as:

0%	-
1-25%	+
26-50%	++
51-75%	+++
76-100%	++++

HA receptor	Function	Distribution on tumour	% epitope expression on tumour
CD44H	Isoform which predominantly binds and internalises HA (Culty et al., 1992)	Expressed on all cells with exception of some stromal cells	++++
CD44v6	Role in cancer unknown, but is often used as a prognostic factor. The higher the expression, the lower the survival probability (Friedrichs et al., 1995)		+
CD44v3	Often over-expressed in breast carcinoma (Friedrichs et al., 1995)		-
RHAMM	Required for transformation and tumour cell invasion (Hall et al., 1995)	Groups of infiltrating tumour cells, with high expression on cells surrounding necrotic areas	+++
ICAM-1	Binds and internalises HA, putative metabolic receptor (McCourt et al., 1994)	Present on stromal cells	++
CEA	A fetal antigen expressed on malignant cells (Haskell, 1990)	Present on all tumour cells	++++

a portion of the 24.5mg/ml MTX stock solution and dissolved overnight with vortexing, to give a final concentration of 21mg/ml. To ensure sterility gentamicin was added to a concentration of 50µg/ml and incubated overnight at 4°C. Following the addition of [^{3}H]methotrexate the HA/MTX stock mixture was diluted to injection concentration with injection grade sodium chloride. Injections were individually made according to mouse body masses, to deliver 15mg/kg MTX and 12.5mg/kg HA in 50µl. With this quantity of HA injected into the body, saturation kinetics would be observed for the period of the experiment (Fraser *et al*, 1983).

To ensure that the HA had maintained its molecular weight during the preparation of the methotrexate/HA injection mixture, the injection solution was analysed on a Sephacryl S-1000 size exclusion gel (Pharmacia, Uppsala, Sweden) with column specifications of 1.6cm x 70cm, sample size 2ml, flow rate 18ml/h and 2ml fraction size. Figure 6 shows that HA retained its molecular weight during the mixing procedure.

Mice were randomly divided into 2 groups of 40 animals. Group I received MTX only, and Group 2 received MTX/HA combination therapy. Animals were individually placed in an injection box, and the injections were administered via the tail vein. Tritiated methotrexate (mean injected disintegration's per minute (dpm) ± standard error of the mean (SEM): 19,159,146 ± 1,336,819) contained within 15mg/kg MTX ± 12.5mg/kg HA was delivered in each injection. Mice were individually housed in soft, non-wettable plastic enclosures so urine could be collected. At 30min, 1h, 2h, 4h or 8h after injection mice were anaesthetised by 0.1ml intra-peritoneal injection of Nembutal (Glaxo, Australia Pty. Ltd., Melbourne, Australia), and blood was collected from the heart or great vessels using a needle and syringe. After blood collection the animals were killed by cervical dislocation.

Blood was delivered into EDTA-coated glass tubes

The amount of MTX delivered to the bloodstream henceforth will be referred to as the "injected dose".

In order to make accurate comparisons between the sample population and normalise slight variations in organ and tumour masses, the concentration of MTX in the body organs and tumour and body fluid was expressed as % of injected dose/gram of tissue.

The mean percentage of the MTX injection remaining at the injection site was 3.78% (SEM: 0.57%). To normalise such variations, the percentage of dpm found in tumour and tissues was calculated as a percentage of the dpm injected minus the dpm found remaining at the injection site. This amount is henceforth referred to as the available dpm or available methotrexate. The results are summarised in Table 2.

No statistically significant difference was noted in the plasma levels of MTX when the drug was co-injected with HA. The gross pharmacokinetics of MTX remained unaltered, with maximum MTX plasma levels reached within 0.5 to 2h following intravenous administration (MIMS, 1997).

When possible urine was collected from the non-wettable plastic enclosures with a syringe and needle. The urine was cleared by centrifugation at 14,000g_{av} for 10 min. Its radioactive content was measured after the addition of 3ml HiSafeII scintillant to samples ranging from 8-30µl. Despite the technical difficulties in accurately quantitating the volume of urine produced by each mouse we calculated the percentage of injected MTX dose in the urine by the following formula:

$$\frac{\text{time of collection (h)} \times 42\mu\text{l} \times \text{dpm}/\mu\text{l urine} \times 100}{\text{Total dpm injected}}$$

= % of injected MTX in urine.

It was not possible to collect urine from each mouse, because of variations in the micturition rate. When 3 or more urine specimens were available per time point per treatment non-parametric statistical analysis of the data at those time points was performed. At one hour after administration there was 50% (p=0.043) more MTX in the urine of mice which received MTX/HA (see Table 2).

Immediately after killing the mouse the tumour, liver, heart, spleen, bladder, left and right kidneys, uterus, lungs, stomach, intestines, brain and lymph nodes were excised and analysed for total radioactivity. The total radioactivity in each tissue was determined by solubilising 100-400mg of tissue in 3-6ml of OptiSolv (ACC, Melbourne, Australia) for 36 h, 22°C. On completion of solubilisation, radioactivity in the tissue was counted after adding 10ml of HiSafeIII scintillant. Again to overcome chemi- and photoluminescence, samples were counted

OptiSolv for 24h at 22°C, followed by the addition of 10ml Hisafe-3 scintillant. To overcome chemi- and photoluminescence, samples were counted for 2min in a Wallac 1410 ß-counter over a 3, 7 or 20 d period depending on the sample source. During the periods between counting, samples were stored in the dark at ambient temperature. All calculations were performed on stabilised samples where all chemi- and photoluminescence had been removed.

The figures represent median ± SEM (n=8). Analysing the data with the non-parametric randomization test for matched pairs demonstrated that the co-administration of HA significantly reduced the excretion of drug into the GI tract ($p=0.031$, one-tailed test).

The decrease in MTX concentration ranged from 43-67%. The non-parametric randomization test for matched pairs showed that the co-administration of HA significantly reduced the excretion of drug into the gastrointestinal tract ($p=0.031$, one-tailed test).

In the lungs there was significantly less MTX present at 4h when co-administered with HA, with a median decrease of 52% ($p=0.014$). No differences were demonstrated at other time points, however, so that the significance of this observation remains uncertain.

No observable trends were detected in the spleen, uterus, brain, heart, lymph nodes, stomach and kidneys.

There are two possible mechanisms of HA targeting of methotrexate to tumour cells (Figure 10).

There was a significant targeting effect when HA was combined with MTX (Figure 7). The greatest relative increase in tumour retention of drug was observed at 0.5h (mean 24% increase), 1h (mean 30% increase) and 2h (mean 119% increase), whereas at 4h and 8h the increase was negligible. Because of the small population size and non-parametric distribution of the data the Mann-Whitney Rank Sum Test test was used, and revealed a significant increase in tumour uptake of drug when HA was co-injected. At 1h the statistical significance was $p=0.021$ and at 2h,

was found in the liver when it was co-injected with HA (4h: 68% less MTX and 8h: 75% less MTX). After intravenous administration MTX is widely distributed in body water, and can be retained in the liver for months (McEvoy, 1988); therefore the decreased median concentration of MTX in the liver at 4 and 8h when co-injected with HA could indicate an altered balance in the routes of pharmacokinetic clearance. Considering that HA is rapidly metabolised within the liver endothelial cells (LEC) it follows that MTX which is co-internalised with HA would be released within the liver sinusoidal lining cells, where it could either diffuse into hepatocytes to be secreted in the bile and subsequently the gastrointestinal tract, or be returned to the circulation for further distribution into body water and for urinary excretion, or both.

There could be a therapeutic advantage of short-term hepatic-targeting. In the case of liver metastasis a rapid, high exposure to MTX could be beneficial, and since the observed targeting is only for 1h this would counteract any long term toxicity problems. Liver targeting could be utilised with drugs which require bio-activation, eg mitomycin C, doxorubicin, where the drug/HA mixture would be targeted to the LEC. With the inactive drug concentrated in the LEC it would be able to diffuse into the hepatocytes for activation, thus acting as an activation targeting mechanism.

One of the major sites of toxicity of MTX is the gastrointestinal tract. Co-administration of MTX with HA significantly diminished the amount of drug delivered to the GI tract. There may be several mechanisms associated with the decreased concentration of MTX in the gut.

Methotrexate is a very small molecule, which one would expect normally to pass through most capillary walls, whereas association with HA would greatly reduce its passage through this route.

Rapid degradation of HA in the liver endothelial cells resulted in a rapid release of MTX into the liver and

as well as potentially reducing the undesired side-effect of gastrointestinal toxicity.

Example 3 **Preparation and injection of paclitaxel/ hyaluronan drug combinations**

Having established the usefulness of the nude mouse model for HA/Paclitaxel, it could now be used to test the effectiveness of other chemotherapeutics. It was decided that, due to its therapeutic importance, paclitaxel (also known as taxol) would be used.

Paclitaxel is isolated from the Western Yew, *Taxus brevifolia*, (Wani *et al* 1971), and is clinically active against advanced ovarian and breast cancer [Rownisky *et al* 1990; McGuire *et al* 1989] and is currently undergoing clinical trials for treatment of a variety of other cancers. However the main problem associated with paclitaxel is its extreme lipophilicity and consequent poor aqueous solubility. Efforts to solve this problem have led to the synthesis of paclitaxel analogues and prodrugs along with extensive efforts to devise safe and biocompatible formulations. To date no prodrugs have shown sufficient stability, solubility or activity that would warrant clinical development (Mathew *et al* 1992; Vyas *et al* 1993). However, semisynthetic taxanes are showing greater solubility and potency than paclitaxel (Bissery *et al* 1991) and one form Taxotere has entered human trials (Bisset *et al* 1993).

The current clinical formulation of paclitaxel employed for intravenous delivery utilises ethanol and Cremophor EL in a 1:1 (v/v) ratio with the drug at 6 mg/mL. Cremophor EL is actually polyethoxylated castor oil; a clear, oily viscous, yellow surfactant. Stability studies have shown that the original formulation has a shelf-life of 5 years at 4°C. The preparation is diluted before use with 0.9% saline or 5% dextrose to concentrations of 0.3-1.2 mg/mL and the physical and chemical stability of the material in these conditions is ca. 27 h. However,

50µl. With this quantity of HA injected into the body, saturation kinetics would be observed for the period of the experiment (Fraser *et al*, 1983).

To ensure that the HA had maintained its molecular weight during the preparation of the paclitaxel/HA injection mixture, the injection solution is analysed on a Sephacryl S-1000 size exclusion gel (Pharmacia, Uppsala, Sweden) with column specifications of 1.6cm x 70cm, sample size 2ml, flow rate 18ml/h and 2ml fraction size. Figure 4 shows that HA retained its molecular weight during the mixing procedure.

Mice are randomly divided into 2 groups of 40 animals. Group I received paclitaxel only, and Group 2 receive paclitaxel/HA combination therapy. Animals are individually placed in an injection box, and the injections are administered via the tail vein. Tritiated paclitaxel (mean injected disintigrations per minute (dpm) ± standard error of the mean (SEM): 19,159,146 ± 1,336,819) is delivered in each injection. Mice are individually housed in soft, non-wettable plastic enclosures so urine can be collected. At 30min, 1h, 2h, 4h or 8h after injection mice are anaesthetised by 0.1ml intra-peritoneal injection of Nembutal (Glaxo, Australia Pty. Ltd., Melbourne, Australia), and blood is collected from the heart or great vessels using a needle and syringe. After blood collection the animals are killed by cervical dislocation.

Blood is delivered into EDTA-coated glass tubes and plasma is prepared by centrifugation at 14,000g_{av} for 10 min. Radioactivity is counted in 50µl aliquots after decolourisation with 100µl of 30%v/v hydrogen peroxide and the addition of 3ml HiSafeII scintillant. To overcome chemi- and photoluminescence, samples are counted for 2min in a Wallac 1410 ß-counter over a 3, 7 or 20 d period, depending on the sample source. During the periods between counting, samples are stored in the dark at ambient temperature. All calculations are performed on stabilised samples from which all chemi- and photoluminescence had

The mean percentage of the paclitaxel injection remaining at the injection site is calculated. To normalise such variations, the percentage of dpm found in tumour and tissues is calculated as a percentage of the dpm injected minus the dpm found remaining at the injection site. This amount is referred to as the available dpm or available paclitaxel.

When possible urine is collected from the non-wettable plastic enclosures with a syringe and needle. The urine is cleared by centrifugation at 14,000g_{av} for 10 min. Its radioactive content is measured after the addition of 3ml HiSafeII scintillant to samples ranging from 8-30µl.

Immediately after killing the mouse the tumour, liver, heart, spleen, bladder, left and right kidneys, uterus, lungs, stomach, intestines, brain and lymph nodes are excised and analysed for total radioactivity. The total radioactivity in each tissue is determined by solubilising 100-400mg of tissue in 3-6ml of OptiSolv (ACC, Melbourne, Australia) for 36 h, 22°C. On completion of solubilisation, radiactivity in the tissue is counted after adding 10ml of HiSafeIII scintillant. Again to overcome chemi- and photoluminescence, samples are counted for 2min in a Wallac 1410 ß-counter over a 3, 7 or 20 d period depending on the sample source. During the periods between counting, samples are stored in the dark at ambient temperature. All calculations are performed on stabilised samples from which all chemi- and photoluminescence had been removed.

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Methotrexate entrained in the hyaluronan meshwork

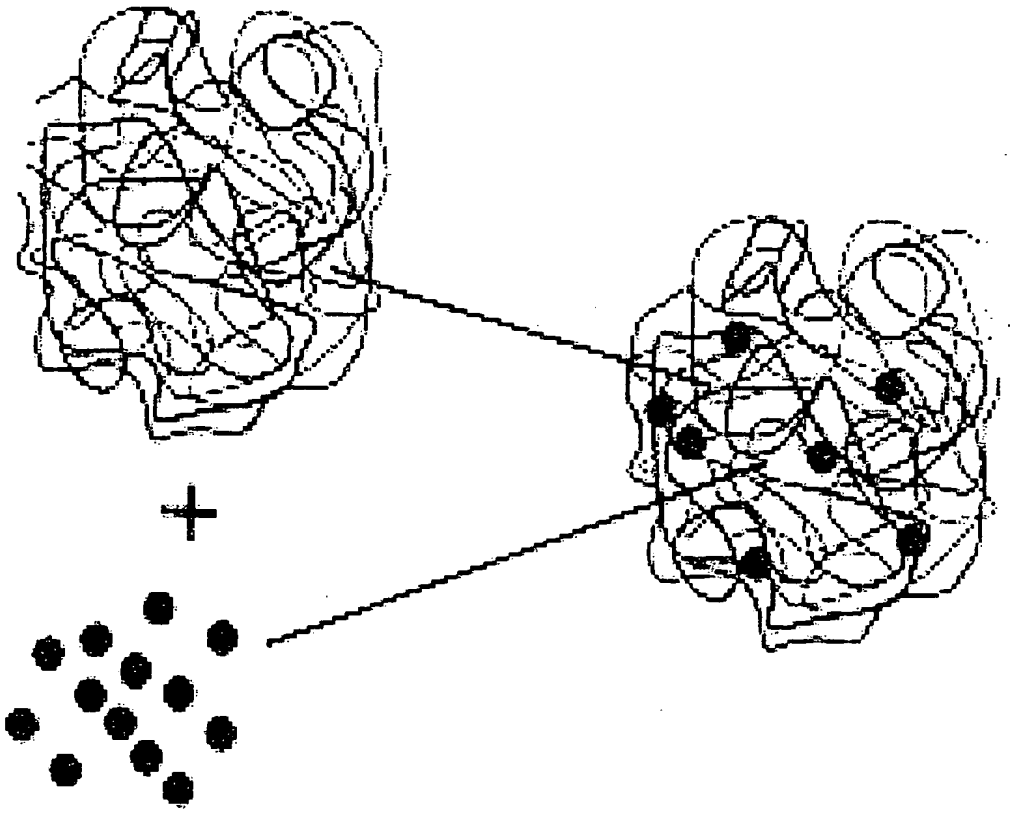
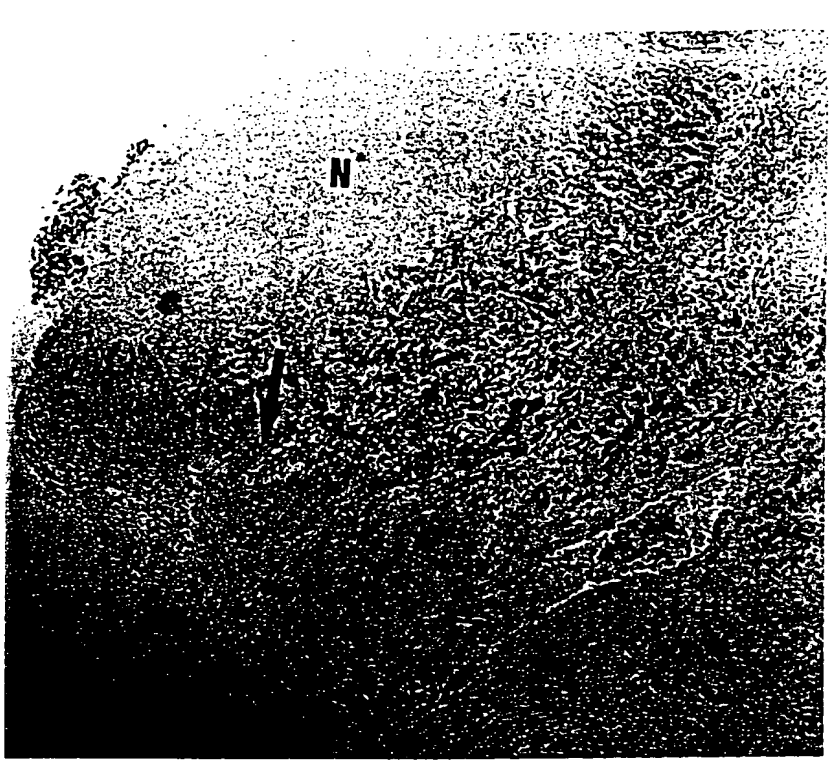


FIGURE 1



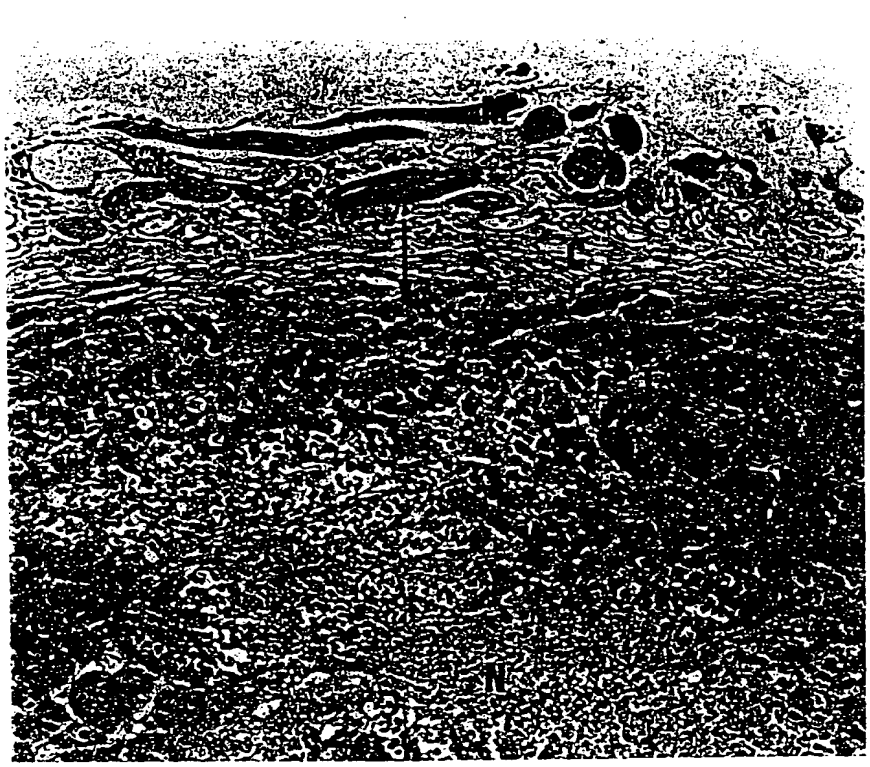


FIGURE 3

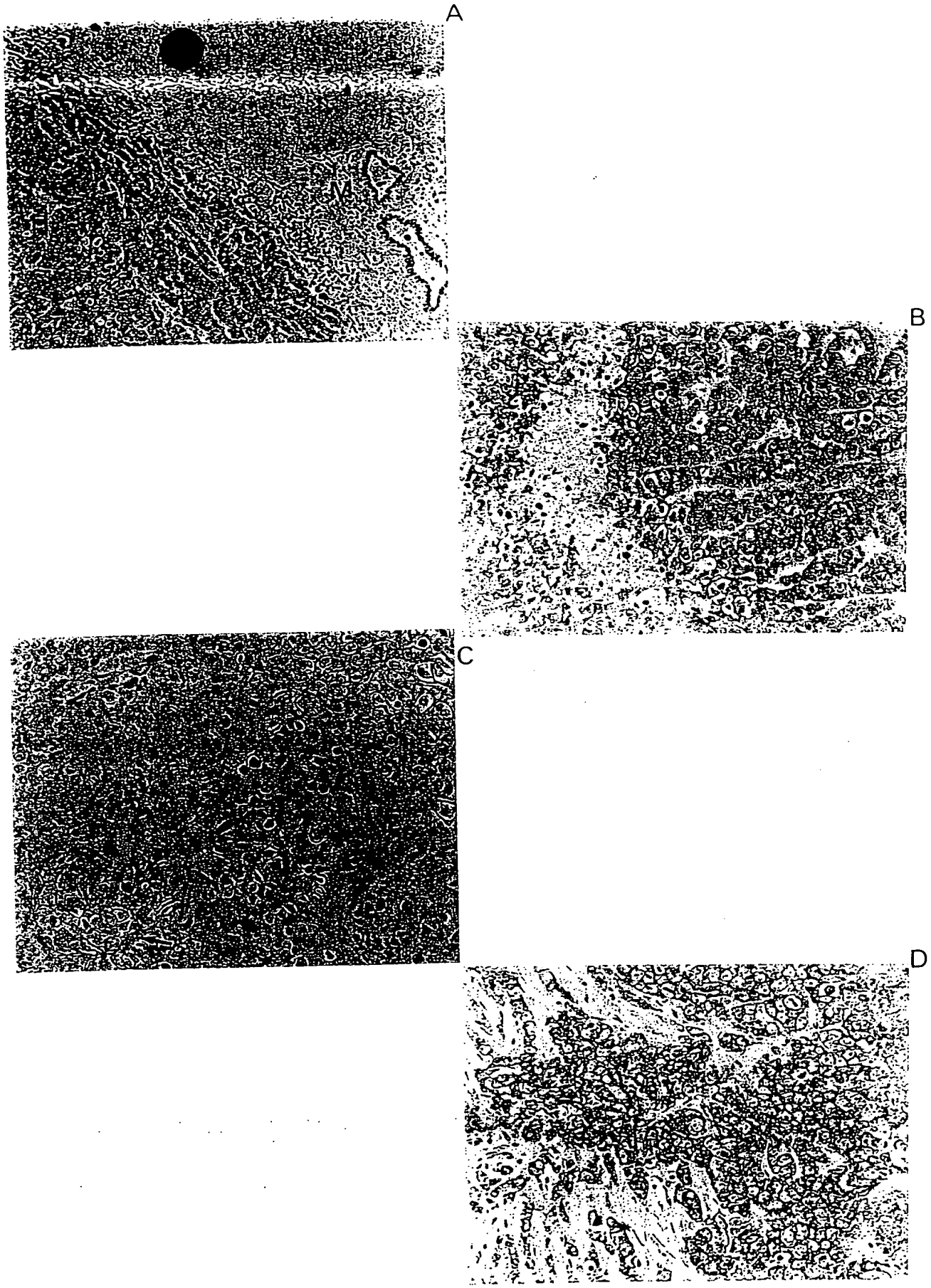


FIGURE 5

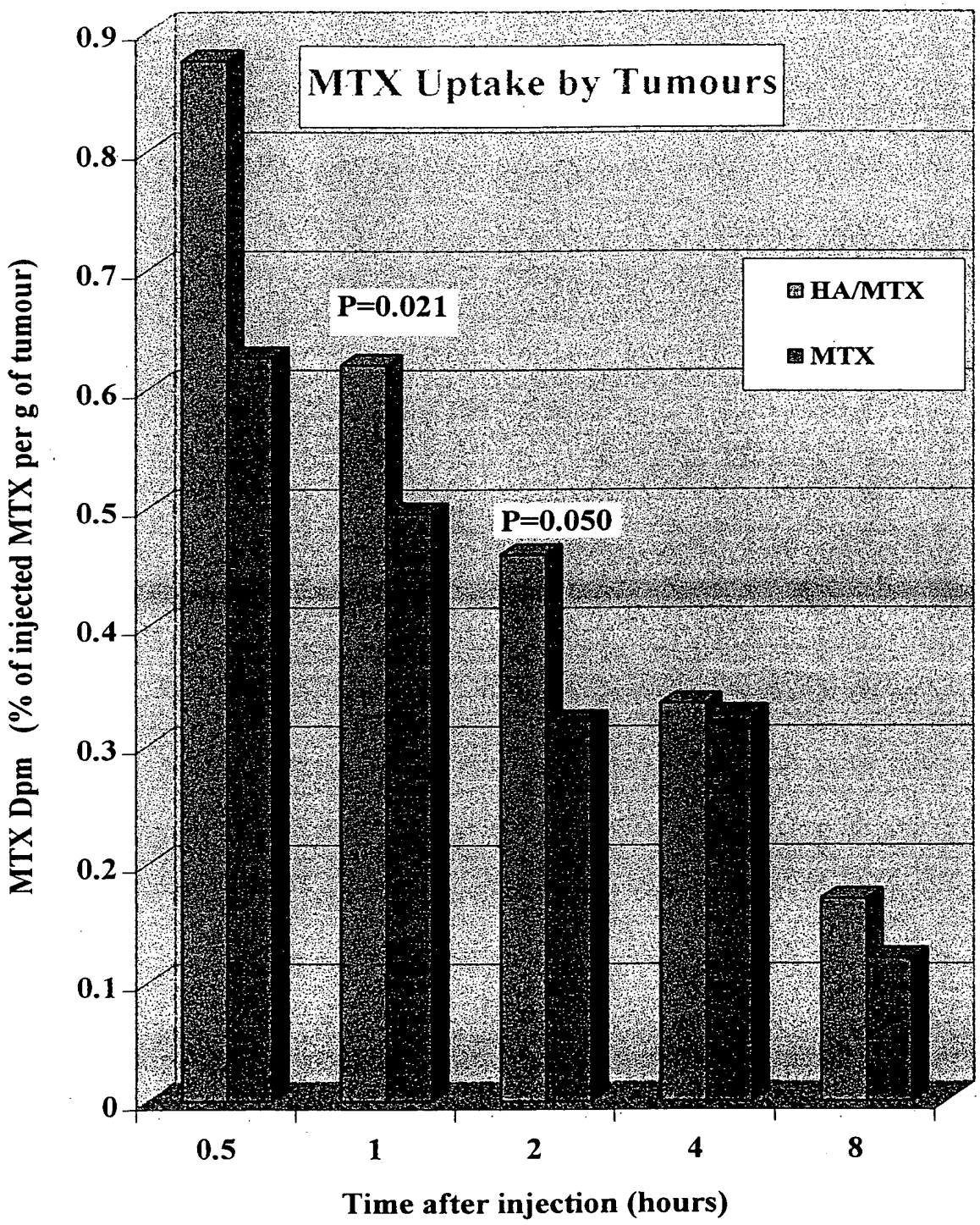

MTX Uptake by Tumours
HA/MTX
MTX
P=0.021
P=0.050
MTX Dpm (% of injected MTX per g of tumour)
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0.5
1
2
4
8
Time after injection (hours)

FIGURE 7

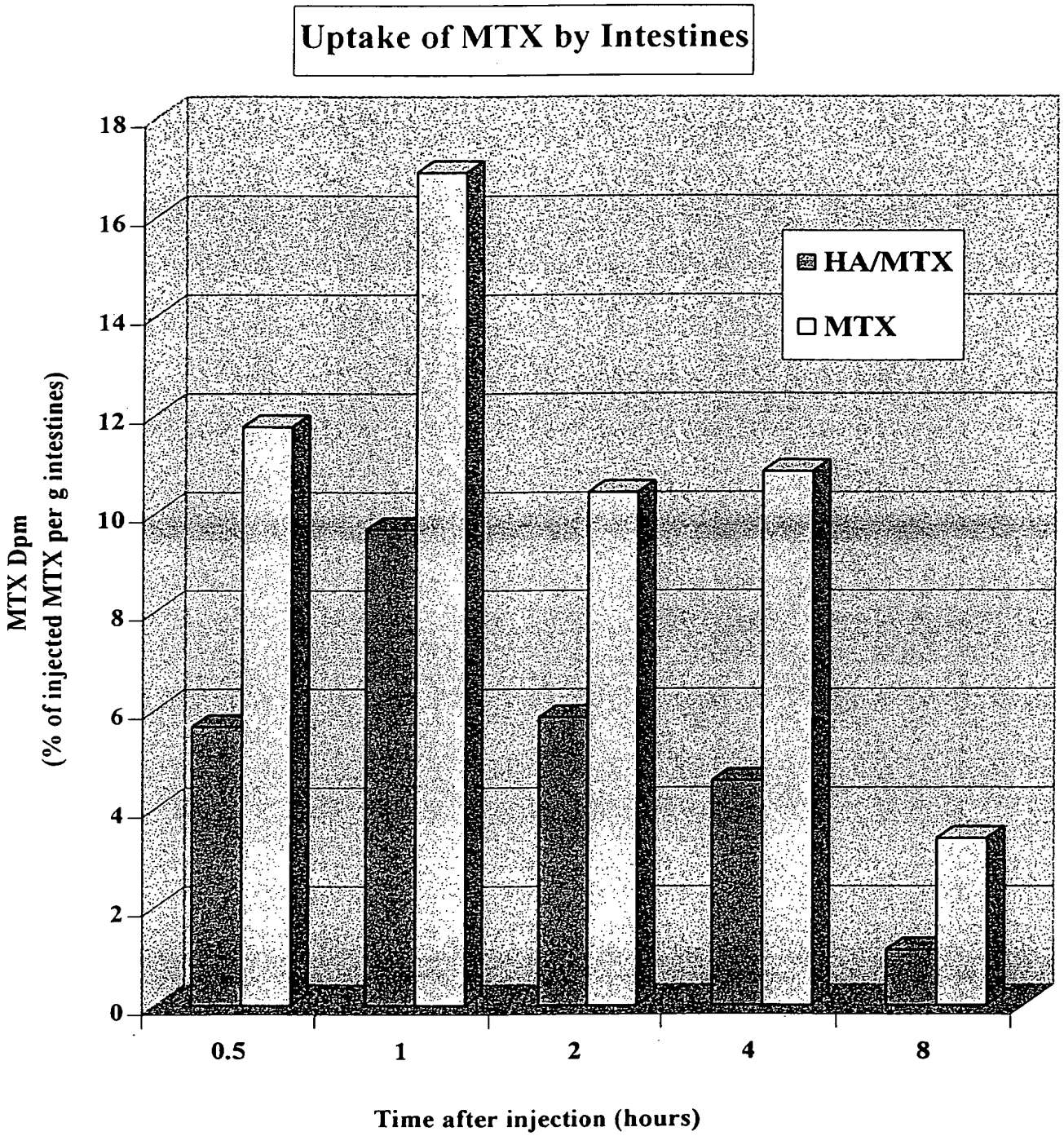

Uptake of MTX by Intestines
MTX Dpm
(% of injected MTX per g intestines)
18
16
14
12
10
8
6
4
2
0
HA/MTX
MTX
0.5
1
2
4
8
Time after injection (hours)

FIGURE 9